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**PATENT APPLICATION**  
**USE OF INSECT CELL MEMBRANE TRANSPORTERS AS NOVEL**  
**TARGET SITES FOR INSECTICIDES**

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## USE OF INSECT CELL MEMBRANE TRANSPORTERS AS NOVEL TARGET SITES FOR INSECTICIDES

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### CROSS-REFERENCES TO RELATED APPLICATIONS

Not Applicable.

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### STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was made with Government support under NIH grant  
AI34524. The government has certain rights to this invention.

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### BACKGROUND OF THE INVENTION

#### Field of the Invention

The present invention relates to a field of insect transporter nucleic acids  
and proteins, and their use as novel target sites for insecticide development and  
insecticide action. Recombinant proteins and peptides with insecticidal activity, isolated  
DNA molecules encoding them, vectors comprising the nucleic acids, and methods of  
preparing them are provided. Methods of screening for compounds that modulate activity  
of or bind to membrane transporters are also provided.

#### Description of the Related Art

At present, insecticide resistance is posing serious problems in pest  
management. Consequently the development of new molecular targets in insect systems  
is greatly increased.

One method of controlling insect development consists of using  
biologically active hormones to interfere with insect developmental processes. This  
method has been used in several insect-based industries, such as in the silk industry. For  
example, JP 79042912 and JP 50029371 (both to Ajinomoto KK) relate to cultivation of  
silkworms by feeding with an ecdysis hormone component and juvenile hormone. JP  
51013684 (to Takeda) also employs biologically active hormones in combination with  
contamination controlling agents, as part of a method for preventing internal silkworm

contamination. This method, more specifically, provides for treatment with steroid-like molting hormone, juvenile hormone, and antibiotics at specific developmental stages.

Approaches such as the one above suffer several disadvantages, most significantly a relatively limited effectiveness because of the narrow window of susceptibility of insects to these types of steroid-like hormones. On a commercial scale, such agents for insecticidal applications are relatively ineffective, providing for disruption of insect development only at the end of a molt episode.

Insects, including lepidopteran insects, continue to elicit significant loss to many commercially important agricultural crops, including grains (corn, wheat, cotton, soybeans), and various vegetable, fruit (grapes, apples, peaches), and nut crops (almonds, walnuts). Hence, significant economic incentive exists for developing safer and more economic insect controlling strategies.

Conventional insect pest control methods rely primarily on relatively toxic, and non-specific chemical formulations, and have become increasingly unacceptable because of potential toxicities to humans and animals, as well as destruction of desirable plant and animal life. The continued threat such agents pose to the environment add to the growing need for more bio-compatible, specific, yet effective, insect population control techniques. Repeated use of conventional chemical insecticides also enhances the potential for insect resistance, resulting in increased risk of insecticide resistant insect strains and reduced effectiveness.

Alternatively, novel targets for insecticide targets can be identified, such as  $\gamma$ -Aminobutyric acid (GABA). GABA is the primary neurotransmitter in the vertebrate central nervous system and in invertebrate central and peripheral nervous systems. For example, in *Manduca sexta*, GABA has been shown to inhibit neuronal activity, suggesting its involvement in regulating central neural functions in this insect. GABA transporters and other transporters affecting the central nervous system of insects could be used to provide new insecticide targets.

Specifically, insecticides presently available act on only a limited number of target sites and increased insecticide resistance has made many of these targets of limited value. Despite recent advances in the understanding of the biology of insects, a need continues to exist in the art of insect management and control for novel target sites.

## SUMMARY OF THE INVENTION

The present invention discloses the existence of novel insect transporters that can be used as targets for screening of new insecticides.

The nucleic acids of this invention encode insect cell membrane transporter polypeptides including acetylcholine transporters, serotonin transporters, proline transporters, glutamate transporters, neurotransmitter transporters encoded by the inebriated gene, orphan transporters, GABA transporters, and LAT transporters. The polypeptides have greater than about 70% amino acid identity to sequences selected from the group consisting of: SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16.

In one preferred embodiment, the nucleic acids of this invention encode polypeptides with amino acid sequences selected from the group consisting of: SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16. In another preferred embodiment, the nucleic acids have nucleotide sequences selected from the group consisting of: SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, and 15.

This invention also provides insect cell membrane transporter polypeptides with greater than 70% amino acid sequence identity to polypeptides with amino acid sequences selected from the group consisting of: SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16. In another embodiment, this invention provides polypeptides with amino acid sequences selected from the group consisting of: SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16.

This invention also provides methods of screening for compounds which modulate the activity of insect cell membrane transporters encoded sequences selected from the group consisting of: SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, and 15 and nucleic acids encoding the amino acid sequences selected from the group consisting of: SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16. The method comprises the steps of contacting a recombinant cell expressing the insect cell membrane transporter with a test compound and determining the ability of the test compound to modulate the activity of the membrane transporter. In preferred embodiments, the cell membrane transporter has an amino acid sequence selected from the group consisting of: SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16. This invention also provides a compound identified by the above method.

In yet another embodiment, this invention provides methods of screening for a compound which binds to an insect cell membrane transporter. The method initially comprises attaching a membrane transporter polypeptide with an amino acid sequence selected from the group consisting of: SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16 to a

solid surface. This polypeptide is then exposed to test compounds or library of compounds and the ability of the compounds to bind to the transporter is measured. This invention also provides a compound identified by the above method.

In yet another embodiment, the invention provides cells comprising  
5 recombinant nucleic acids encoding cell membrane transporter polypeptides with greater than 70% amino acid identity to sequences selected from the group consisting of: SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16; recombinant nucleic acids with sequences selected from the group consisting of: SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, and 15; and recombinant nucleic acids encoding polypeptides with amino acid sequences selected from the group  
10 consisting of: SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16.

#### BRIEF DESCRIPTION OF SEQUENCE IDENTIFIERS

SEQ ID NO: 1 refers to the nucleotide sequence of the *Manduca sexta*  
15 acetylcholine transporter.

SEQ ID NO: 2 refers to the amino acid sequence of the *Manduca sexta* acetylcholine transporter.

SEQ ID NO: 3 refers to the nucleotide sequence of the *Manduca sexta* serotonin transporter.

20 SEQ ID NO: 4 refers to the amino acid sequence of the *Manduca sexta* serotonin transporter.

SEQ ID NO: 5 refers to the nucleotide sequence of the *Manduca sexta* proline transporter.

25 SEQ ID NO: 6 refers to the amino acid sequence of the *Manduca sexta* proline transporter.

SEQ ID NO: 7 refers to the nucleotide sequence of the *Aedes aegypti* glutamate transporter.

SEQ ID NO: 8 refers to the amino acid sequence of the *Aedes aegypti* glutamate transporter.

30 SEQ ID NO: 9 refers to the nucleotide sequence of the *Manduca sexta* neurotransmitter transporter encoded by the inebriated gene.

SEQ ID NO: 10 refers to the amino acid sequence of the *Manduca sexta* neurotransmitter transporter encoded by the inebriated gene.

SEQ ID NO: 11 refers to the nucleotide sequence of the *Manduca sexta* orphan transporter.

SEQ ID NO:12 refers to the amino acid sequence of the *Manduca sexta* orphan transporter.

5 SEQ ID NO: 13 refers to the nucleotide sequence of the *Manduca sexta* GABA transporter.

SEQ ID NO: 14 refers to the amino acid sequence of the *Manduca sexta* GABA transporter.

10 LAT transporter.  
SEQ ID NO: 15 refers to the nucleotide sequence of the *Aedes aegypti*

SEQ ID NO: 16 refers to the amino acid sequence of the *Aedes aegypti* LAT transporter.

## 15 DESCRIPTION OF THE SPECIFIC EMBODIMENTS

### I. Definitions

“Insect cell membrane transporter” refers to a gene, or the protein it encodes, that in its wildtype form has the ability to transport a compound across an insect cell membrane. If an insect cell membrane transporter gene is damaged (e.g., by radiation, a carcinogen or inherited, or spontaneous mutation) or blocked from functioning (e.g., by specifically binding to another substance other than the one normally transported), it may lose its wildtype ability to transport compounds across the cell membrane. Preferred transporters include but are not limited to transporters that are present in the nervous system, alimentary canal or malpighian tubules of insects, for example, a proline transporter, an acetylcholine transporter, a serotonin transporter, a glutamate transporter, acetylcholine transporter, a neurotransmitter transporter encoded by the inebriated gene, a GABA transporter, and a LAT transporter.

The term “insect cell membrane transporter” also refers to polymorphic variants, alleles, interspecies homologs, and mutants that: (1) have about 70% amino acid sequence identity, preferably about 80-90% amino acid sequence identity to SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, or 16 over a window of about at least 50-100 amino acids; (2) binds to polyclonal antibodies raised against an immunogen comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16 and conservatively modified variants thereof; (3) specifically hybridize (with a size of

at least about 500, preferably at least about 900 nucleotides) under stringent hybridization conditions to a sequence selected from the group consisting of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, and 15 and conservatively modified variants thereof; or (4) are amplified by primers that specifically hybridize under stringent conditions to the same sequence as

5 degenerate primers, including, but not limited to, the following:

1S forward primer for NTTs: (A69)

5'-CGGAATTCTGG(G/C)CAA(T/C)(G/A)TITGG(A/C)GITT(C/T)CCNTA-3'

4A reverse primer for NTTs: (A67)

10 5'-GCCAAGCTTGAAGAAGAT(C/T)TG(G/A)GIIGCIGC(G/A)TCNA(C/T/G)CCA-3'

2S reverse primer for NTTs: (A70)

C-TCC-ATG-GA(AG)-AA(TC)-GGI-GGI-GGI-GCN-TT

15 3A reverse primer for NTTs: (A68)

GGC-GAG-CTC-GGC-ICC-IGG-IAG-IGT-N(AG)C-NCC

“Insecticide” refers to an agent, formulation, or preparation that destroys or controls insects, or is hostile or repellant to insects.

20 The terms “isolated,” “purified,” or “biologically pure” refer to material that is substantially or essentially free from components which normally accompany it as found in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated insect cell membrane transporter nucleic acid is separated from open reading frames that flank the insect cell membrane transporter gene and encode proteins other than insect cell membrane transporters. The term “purified” denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

“Nucleic acid” refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or

linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

The terms “polypeptide,” “peptide,” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an analog or mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

The term “amino acid” refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline,  $\gamma$ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an  $\alpha$  carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group (*e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium). Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refer to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

“Conservatively modified variants” applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively



modified variants refer to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are “silent variations,” which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alter, add or delete a single amino acid or a small percentage of amino acids in the encoded sequence is a “conservatively modified variant” where the alteration results in the substitution of an amino acid with a chemically similar amino acid.

Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

The following groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Glycine (G);
- 2) Serine (S), Threonine (T);
- 3) Aspartic acid (D), Glutamic acid (E);
- 4) Asparagine (N), Glutamine (Q);
- 5) Cysteine (C), Methionine (M);

- 6) Arginine (R), Lysine (K), Histidine (H);
- 7) Isoleucine (I), Leucine (L), Valine (V); and
- 8) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

(see, e.g., Creighton, *Proteins* (1984)).

5           An “expression vector” is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

10           The phrase “selectively (or specifically) hybridizes to” refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (e.g., total cellular or library DNA or RNA).

15           The phrase “stringent hybridization conditions” refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic*  
20 *Probes*, “Overview of principles of hybridization and the strategy of nucleic acid assays” (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength pH. The  $T_m$  is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at  
25 equilibrium (as the target sequences are present in excess, at  $T_m$ , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes  
30 (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as

following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with a wash in 0.2x SSC, and 0.1% SDS at 65°C.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary “moderately stringent hybridization conditions” include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

A further indication that two polynucleotides are substantially identical is if the reference sequence, amplified by a pair of oligonucleotide primers or a pool of degenerate primers that encode a conserved amino acid sequence, can then be used as a probe under stringent hybridization conditions to isolate the test sequence from a cDNA or genomic library, or to identify the test sequence in, *e.g.*, a Northern or Southern blot. Alternatively, another indication that the sequences are substantially identical is if the same set of PCR primers can be used to amplify both sequences.

The terms “identical” or percent “identity,” or “sequence identity” in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

The phrase “substantially identical,” in the context of two nucleic acids or polypeptides, refers to two or more sequences or subsequences that have at least 60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In a

most preferred embodiment, the sequences are substantially identical over the entire length of the coding regions.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (*see generally, Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1995 Supplement) (Ausubel)).

Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-410 and Altschuel *et al.* (1977) *Nucleic Acids Res.* 25: 3389-3402, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al., supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a

scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described below.

“Antibody” refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one “light” (about 25 kDa) and one “heavy” chain (about 50-70 kDa). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains respectively.

Antibodies exist, *e.g.*, as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)<sub>2</sub>, a dimer of Fab which itself is a light chain joined to VH-CH1 by a disulfide bond. The F(ab)<sub>2</sub> may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab)<sub>2</sub> dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see Fundamental Immunology* (Paul ed., 3<sup>rd</sup> ed. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA methodologies (*e.g.*, single chain Fv).

For preparation of monoclonal or polyclonal antibodies, any technique known in the art can be used (*see, e.g.*, Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor *et al.*, *Immunology Today* 4: 72 (1983); Cole *et al.*, pp. 77-96 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985)). Techniques for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies. Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (*see, e.g.*, McCafferty *et al.*, *Nature* 348:552-554 (1990); Marks *et al.*, *Biotechnology* 10:779-783 (1992)).

The phrase “selectively associates with” refers to the ability of a nucleic acid to “selectively hybridize” with another as defined above, or the ability of a test compound to “selectively (or specifically) bind to an insect cell membrane transporter, as defined above.

The term “recombinant” when used with reference, *e.g.*, to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

“Test compound” refers to any chemical compound, synthetic or naturally occurring, for example small organic molecules, peptides, or antisense nucleic acids. In the present context, such compounds are screened for ability to bind to or modulate the activity of an insect cell membrane transporter. Such compounds can be used to formulate insecticides for the destruction or control of insect populations.

“Modulates activity” refers to the ability of a compound to inhibit, activate, modulate or bind to an insect cell transporter. Such activity can be tested and measured by methods known to one of skill in the art.

The phrase “functional effects” in the context of assays for testing compounds that modulate activity of an insect cell membrane transporter includes the determination of any parameter that is indirectly or directly under the influence of the insect cell membrane transporter protein. Functional effects include, *e.g.*, increase in flux across the membrane, decrease in flux across the membrane.

By “determining the functional effect” is meant assays for a compound that increases or decreases a parameter that is directly or indirectly under the influence of an insect cell membrane transporter. Such functional effects can be measured by any means known to those skilled in the art.

“Inhibitors,” “activators,” and “modulators” of insect cell membrane transporter activity refer to inhibitory, activating, or modulatory molecules identified using *in vitro* and *in vivo* assays for membrane transport, *e.g.*, ligands, agonists, antagonists, and their homologs and mimetics. Inhibitors are compounds that decrease, block, prevent, delay activation, or inactivate membrane transport, *e.g.*, antagonists. Activators are compounds that increase, activate, facilitate, or enhance activation of transporters, *e.g.*, agonists. Modulators are inhibitors and activators and include genetically modified versions of insect cell membrane transporters, *e.g.*, with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, small chemical molecules and the like. Such assays for modulators include, *e.g.*, expressing an

insect cell membrane transporter in cells, applying putative modulator compounds, and then determining the functional effects on inhibition of membrane transport. Compounds identified by these assays are used in insecticide preparations.

Samples or assays comprising an insect cell membrane transporter that has been treated with a potential modulator are compared to control samples without the inhibitor, activator, or modulator. Control samples (untreated with inhibitors) are assigned a relative transport activity value of 100%. Inhibition of transport is achieved when the insect cell membrane transporter activity value relative to the control is about 90%, preferably 50%, more preferably 250%. Activation of the transporter is achieved when the insect cell membrane transporter activity value relative to the control (untreated with activators) is 110%, more preferably 150%, more preferably 200-500%, more preferably 1000-3000% higher.

## II. Isolation of genes encoding insect transporters

The present invention discloses the existence of novel insect transporters that can be used as targets for screening of new insecticides. The nucleic acids encoding the transporter targets disclosed here were isolated from *Manduca Sexta* 5<sup>th</sup> instar larvae CNS, *Manduca Sexta* embryo, and *Aedes aegypti* midgut and malpighian tubules. Elucidation of the nucleic acid and amino acid sequences of these proteins and the existence of tissues/glands homologous to those used herein in many other insects and animals, provide a number of different natural sources from which these targets may be derived. Insect cell transporters are present in many insect tissues. One embodiment focuses on the transporters present in the nervous system, alimentary canal and in malpighian tubules. For example, conserved transporter sequences for use as primers are optionally identified from mammals and *C. elegans*.

### A. General recombinant DNA methods

Insect transporter polypeptides and nucleic acids are used in the assays described below. For example, the nucleic acids and proteins can be used to identify novel insecticides and to provide target sites for insecticide action. Such polypeptides and nucleic acids can be made using routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2<sup>nd</sup> ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)).



For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

Oligonucleotides can be chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Letts.* 22:1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et al.*, *Nucleic Acids Res.* 12:6159-6168 (1984). Purification of oligonucleotides is typically by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* 255:137-149 (1983). The sequence of the cloned genes and synthetic oligonucleotides can be verified after cloning using, *e.g.*, the chain termination method for sequencing double-stranded templates of Wallace *et al.*, *Gene* 16:21-26 (1981). Again, as noted above, companies such as Operon Technologies, Inc. provide an inexpensive commercial source for essentially any oligonucleotide.

#### *B. Cloning methods for the isolation of nucleotide sequences encoding insect membrane transporters*

In general, the nucleic acid sequences encoding genes of interest, such as sequences for the GABA, proline, serotonin, acetylcholine, glutamate transporters and the neurotransmitter transporter encoded by the inebriated gene, GABA transporters, and LAT transporters, as well as related nucleic acid sequence homologs, are cloned from cDNA and genomic DNA libraries by hybridization with a probe, or isolated using amplification techniques with oligonucleotide primers. Preferably insect sequences are used. For example, acetylcholine transporter sequences are typically isolated from insect nucleic acid (genomic or cDNA) libraries by hybridizing with a nucleic acid probe, the sequence of which can be derived from SEQ ID NO: 1. A suitable tissue from which insect membrane transporter RNA and cDNA can be isolated is, *e.g.*, *Manduca sexta* embryo tissue, such as brain or ventral nerve cord tissue.

Amplification techniques using primers can also be used to amplify and isolate, *e.g.*, a nucleic acid encoding the GABA or serotonin transporter, from DNA or RNA (*see, e.g.*, Dieffenbach & Dveksler, *PCR Primer: A Laboratory Manual* (1995)).

These primers can be used, *e.g.*, to amplify either the full length sequence or a probe of one to several hundred nucleotides, which is then used to screen a mammalian library for the full-length nucleic acid of choice. For example, degenerate primer sets, can be used to isolate insect cell membrane transporter nucleic acids. Nucleic acids can also be isolated from expression libraries using antibodies as probes. Such polyclonal or monoclonal antibodies can be raised, *e.g.*, using the sequence of an insect cell membrane transporter, such as the GABA transporter or proline transporter.

Polymorphic variants and alleles that are substantially identical to the gene of choice can be isolated using nucleic acid probes, and oligonucleotides under stringent hybridization conditions, by screening libraries. Alternatively, expression libraries can be used to clone, *e.g.*, acetylcholine transporters and proline transporters, polymorphic variants, interspecies homologs, and alleles, by detecting expressed homologs immunologically with antisera or purified antibodies made against a specific transporter of interest, such as the serotonin transporter, which also recognizes and selectively binds to a serotonin transporter homolog.

To make a cDNA library, one should choose a source that is rich in the mRNA of choice, *e.g.*, for the transporters of the invention embryo tissue from *M. sexta* is optionally used. The mRNA is then made into cDNA using reverse transcriptase, ligated into a recombinant vector, and transfected into a recombinant host for propagation, screening and cloning. Methods for making and screening cDNA libraries are well known (*see, e.g.*, Gubler & Hoffman, *Gene* 25:263-269 (1983); Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*).

For a genomic library, the DNA is extracted from the tissue and either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb. The fragments are then separated by gradient centrifugation from undesired sizes and are constructed in non-lambda expression vectors. These vectors are packaged *in vitro*. Recombinant phage are analyzed by plaque hybridization as described in Benton & Davis, *Science* 196:180-182 (1977). Colony hybridization is carried out as generally described in Grunstein *et al.*, *Proc. Natl. Acad. Sci. USA.*, 72:3961-3965 (1975).

An alternative method of isolating a nucleic acid and its homologs combines the use of synthetic oligonucleotide primers and amplification of an RNA or DNA template (*see* U.S. Patents 4,683,195 and 4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Methods such as polymerase chain reaction (PCR) and ligase chain reaction (LCR) can be used to amplify nucleic acid

sequences of, *e.g.*, serotonin and proline transporters, directly from mRNA, from cDNA, from genomic libraries, or cDNA libraries. Degenerate oligonucleotides can be designed to amplify transporter homologs using the sequences provided herein. Restriction endonuclease sites can be incorporated into the primers. Polymerase chain reaction or other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of transporters encoding mRNA in physiological samples, for nucleic acid sequencing, or for other purposes. Genes amplified by the PCR reaction can be purified from agarose gels and cloned into an appropriate vector.

As described above, gene expression of membrane transporters can also be analyzed by techniques known in the art, *e.g.*, reverse transcription and PCR amplification of mRNA, isolation of total RNA or poly A+ RNA, northern blotting, dot blotting, *in situ* hybridization, RNase protection, probing high density oligonucleotides, and the like. All of these techniques are standard in the art.

Synthetic oligonucleotides can be used to construct recombinant genes for use as probes or for expression of protein. This method is performed using a series of overlapping oligonucleotides usually 40-120 bp in length, representing both the sense and non-sense strands of the gene. These DNA fragments are then annealed, ligated and cloned. Alternatively, amplification techniques can be used with precise primers to amplify a specific subsequence of a transporter nucleic acid. The specific subsequence is then ligated into an expression vector.

The nucleic acid encoding the protein of choice is typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryote vectors, *e.g.*, plasmids, or shuttle vectors. Optionally, cells can be transfected with recombinant transporter nucleic acids operably linked to a constitutive promoter, to provide higher levels of transporter expression in cultured cells.

### *C. Expression in prokaryotes and eukaryotes*

To obtain high level expression of a cloned gene or nucleic acid, such as those cDNAs encoding the cell membrane transporters of the invention, one typically subclones the transporter of interest, *e.g.*, the acetylcholine or serotonin transporter, into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and if for a nucleic acid encoding a protein, a

ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, *e.g.*, in Sambrook *et al.* and Ausubel *et al.* Bacterial expression systems for expressing the transporter proteins are available in, *e.g.*, *E. coli*, *Bacillus sp.*, and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983)). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. For example, the GABA transporter is optionally expressed in *Xenopus* oocytes.

The promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function. The promoter typically also includes elements that are responsive to transactivation, *e.g.*, hypoxia responsive elements, Gal4 responsive elements, lac repressor responsive elements, and the like. The promoter can be constitutive or inducible, heterologous or homologous.

In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the nucleic acid in host cells. A typical expression cassette thus contains a promoter operably linked, *e.g.*, to the nucleic acid sequence encoding the transporter of interest, and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, *e.g.*, c-myc.

Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, *e.g.*, SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene amplification are also suitable, such as using a baculovirus vector in insect cells, with a transporter encoding sequence under the direction of the polyhedrin promoter or other strong baculovirus promoters.

The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are preferably chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of protein, which are then purified using standard techniques (*see, e.g.*, Colley *et al.*, *J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification, in Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g.*, Morrison, *J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983).

Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host

cell (*see, e.g., Sambrook et al., supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the protein of choice.

After the expression vector is introduced into the cells, the transfected cells are cultured under conditions favoring expression of the cell membrane transporter protein, which is recovered from the culture using standard techniques identified below.

### III. Purification of insect cell membrane transporter peptides

If necessary, naturally occurring or recombinant proteins can be purified for use in functional assays, *e.g.*, to make antibodies to detect transporters, or to use in screening applications as discussed below. Naturally occurring cell membrane transporters, *e.g.*, proline, serotonin, glutamate, or acetylcholine transporters, are purified, *e.g.*, from tissue such as the brain, central nervous system, gut, embryo or even whole insects or any other source of a transporter homolog. Recombinant transporters are purified from any suitable expression system, *e.g.*, by expressing a serotonin transporter in *E. coli* and then purifying the recombinant protein via affinity purification, *e.g.*, by using antibodies that recognize a specific epitope on the protein or on part of the fusion protein, or by using glutathione affinity gel, which binds to GST. In some embodiments, the recombinant protein is a fusion protein, *e.g.*, with GST or Gal4 at the N-terminus.

The protein of choice may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (*see, e.g., Scopes, Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al., supra*; and Sambrook *et al., supra*).

A number of procedures can be employed when recombinant protein is being purified. For example, proteins having established molecular adhesion properties can be reversibly fused to a cell membrane transporter protein, such as proline or acetylcholine transporters. With the appropriate ligand, the cell membrane transporter of interest can be selectively adsorbed to a purification column and then freed from the column in a relatively pure form. The fused protein is then removed by enzymatic activity. Finally, the transporters of interest could be purified using immunoaffinity columns.

*A. Purification of cell membrane transporters from recombinant bacteria*

Recombinant proteins are expressed by transformed bacteria in large amounts, typically after promoter induction. Promoter induction with IPTG is one example of an inducible promoter system. Bacteria are grown according to standard procedures in the art. Fresh or frozen bacteria cells are used for isolation of protein.

Proteins expressed in bacteria may form insoluble aggregates (“inclusion bodies”). Several protocols are suitable for purification of inclusion bodies. For example, purification of inclusion bodies typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, *e.g.*, by incubation in a buffer of 50 mM TRIS/HCL pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM ATP, and 1 mM PMSF. The cell suspension can be lysed using 2-3 passages through a French press, homogenized using a Polytron (Brinkman Instruments) or sonicated on ice. Alternate methods of lysing bacteria are apparent to those of skill in the art (*see, e.g., Sambrook et al., supra; Ausubel et al., supra*).

If necessary, the inclusion bodies are solubilized, and the lysed cell suspension is typically centrifuged to remove unwanted insoluble matter. Proteins that formed the inclusion bodies may be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents which are capable of solubilizing aggregate-forming proteins, for example SDS (sodium dodecyl sulfate), 70% formic acid, are inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of activity. Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of biologically active protein. Other suitable buffers are known to those skilled in the art. The protein of choice is separated from other bacterial proteins by standard separation techniques, *e.g.*, with Ni-NTA agarose resin.

Alternatively, it is possible to purify the recombinant transporter protein from bacteria periplasm. After lysis of the bacteria, when the protein is exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to skill in the art. To isolate recombinant proteins from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells,

the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM MgSO<sub>4</sub> and kept in an ice bath for approximately 10 minutes. The cell suspension is centrifuged and the supernatant decanted and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

*B. Standard protein separation techniques for purifying cell membrane transporters*

Solubility fractionation

Often as an initial step, particularly if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol includes adding saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This concentration will precipitate the most hydrophobic of proteins. The precipitate is then discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

Size differential filtration

The molecular weight of the cell membrane transport protein, *e.g.*, GABA, proline, or serotonin transporters, can be used to isolated it from proteins of greater and lesser size using ultrafiltration through membranes of different pore size (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular



weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

#### Column chromatography

5                   The cell membrane transporter protein of choice can also be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art. It will be apparent to one of skill that chromatographic techniques can be  
10 performed at any scale and using equipment from many different manufacturers (*e.g.*, Pharmacia Biotech).

#### **IV. Immunological detection of cell membrane transporters**

                  In addition to the detection of cell membrane transporter genes and gene  
15 expression using nucleic acid hybridization technology, one can also use immunoassays to detect the cell membrane transporters of the invention, *e.g.*, to identify alleles, mutants, polymorphic variants and interspecies homologs of insect cell membrane transporters. Immunoassays can be used to qualitatively or quantitatively analyze transporters, *e.g.*, to detect the presence of a transporter protein, to measure transporter activity, or to identify  
20 modulators of transporter activity. A general overview of the applicable technology can be found in Harlow and Lane, *Antibodies: A Laboratory Manual* (1988).

##### *A. Antibodies to insect cell membrane transporters*

                  Methods of producing polyclonal and monoclonal antibodies that react  
25 specifically with the cell membrane transporters of the invention, *e.g.*, SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16 are known to those of skill in the art (*see, e.g.*, Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *supra*; Goding, *Monoclonal Antibodies: Principles and Practice* (2<sup>nd</sup> ed. 1986); and Kohler & Milstein, *Nature* 256:495-497 (1975). Such techniques include antibody preparation by selection of  
30 antibodies from libraries of recombinant antibodies in phage or similar vectors, as well as preparation of polyclonal and monoclonal antibodies by immunizing rabbits or mice (*see, e.g.*, Huse *et al.*, *Science* 246:1275-1281 (1989); Ward *et al.*, *Nature* 341:544-546 (1989)). In addition, as noted above, many companies, such as BMA Biomedicals, Ltd.,

HTI Bio-products, and the like, provide the commercial service of making an antibody to essentially any peptide.

A number of cell membrane transporter comprising immunogens may be used to produce antibodies specifically reactive with particular insect cell membrane transporters. For example, recombinant proline or serotonin transporters, or antigenic fragments thereof, are isolated as described herein. Recombinant protein can be expressed in eukaryotic or prokaryotic cells as described above, and purified as generally described above. Recombinant protein is the preferred immunogen for the production of monoclonal or polyclonal antibodies. Alternatively, a synthetic peptide derived from the sequences disclosed herein and conjugated to a carrier protein can be used as an immunogen. Naturally occurring protein may also be used either in pure or impure form. The product is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies may be generated, for subsequent use in immunoassays to measure the protein.

Methods of production of polyclonal antibodies are known to those of skill in the art. To improve reproducibility, an inbred strain of mice (*e.g.*, BALB/C mice) can be immunized to make the antibody; however, standard animals (mice, rabbits, etc.) used to make antibodies are immunized with the protein using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol (*see* Harlow & Lane, *supra*). The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the protein of choice. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired (*see* Harlow & Lane, *supra*).

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (*see* Kohler & Milstein, *Eur. J. Immunol.* 6:511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen.

Monoclonal antibodies and polyclonal sera are collected and titered against the immunogen protein in an immunoassay, for example, a solid phase

immunoassay with the immunogen immobilized on a solid support. Typically, polyclonal antisera with a titer of  $10^4$  or greater are selected and tested for their cross reactivity against non-cell membrane transporter proteins or even other related proteins, *e.g.*, from other organisms, using a competitive binding immunoassay. Specific polyclonal antisera and monoclonal antibodies will usually bind with a  $K_D$  of at least about 0.1 mM, more usually at least about 1  $\mu$ M, preferably at least about 0.1  $\mu$ M or better, and most preferably, 0.01  $\mu$ M or better.

Once cell membrane transporter specific antibodies are available, these proteins can be detected by a variety of immunoassay methods. For a review of immunological and immunoassay procedures, *see Basic and Clinical Immunology* (Stites & Terr eds., 7<sup>th</sup> ed. 1991). Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in *Enzyme Immunoassay* (Maggio, ed., 1980); and Harlow & Lane, *supra*.

#### *B. Immunological binding assays*

Cell membrane transporters, such as GABA or acetylcholine transporters, can be detected and/or quantified using any of a number of well recognized immunological binding assays (*see, e.g.*, U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, *see also Methods in Cell Biology: Antibodies in Cell Biology*, volume 37 (Asai, ed. 1993); *Basic and Clinical Immunology* (Stites & Terr, eds., 7<sup>th</sup> ed. 1991). Immunological binding assays (or immunoassays) typically use an antibody that specifically binds to a protein or antigen of choice (in this case a cell membrane transporter, or antigenic fragments thereof). The antibody may be produced by any of a number of means well known to those of skill in the art and as described above.

Immunoassays also often use a labeling agent to specifically bind to and label the complex formed by the antibody and antigen. The labeling agent may itself be one of the moieties comprising the antibody/antigen complex. Thus, the labeling agent may be a labeled cell membrane transporter polypeptide or a labeled anti-membrane transporter antibody. The labeling agent can be modified with a detectable moiety, such as biotin, to which another molecule can specifically bind, such as streptavidin. A variety of detectable moieties are well known to those skilled in the art.

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to

several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, antigen, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

## V. Screening assays for compounds that modulate transporter activity

Insect cell membrane transporters and their alleles, interspecies homologs, and polymorphic variants participate in transport of a wide variety of molecules across cell membranes. For example, GABA, which is present in various regions of central nervous systems, has been shown to inhibit neuronal activity in *M. sexta* antennal lobes, suggesting its involvement in regulating central nervous function in this insect.

Therefore, compounds that activate or inhibit the GABA transporter action would affect regulation of the central nervous system. Similarly, inhibition of the serotonin transporter would affect nervous system function and consequently insect activity. Any of these changes in transport across cell membranes can be assessed by using a variety of *in vitro* and *in vivo* assays, e.g., stable or transient cell lines expressing the transporter, labeled neurotransmitters, or electrophysiology.

Furthermore, these assays can be used to screen for activators, inhibitors, and modulators of transporter activity. Such activators, inhibitors, and modulators of transporter activity can then be used in insecticides. For example, compounds such as Nipecotnic acid, L-DABA,  $\beta$ -alanine, BABA, ACHC, and hemicholinium, guvacine hydrochloride, and cocaine hydrochloride can be prepared in a buffer, such as (Buffer B), and introduced into the transport chamber as part of an assay for detecting transporter inhibition or activation. Compounds that are of interest include analogs of neurotransmitters, natural products, venoms and those from combinatorial libraries. Compounds found to inhibit a transport function necessary, e.g., for central nervous system function, are then optionally used in insecticide formulations.

Biologically active or inactivated transporter polypeptides, either recombinants or naturally occurring, are used to screen for activators, inhibitors, or modulators of transport mechanisms. The transporter polypeptides can be recombinantly expressed in a cell, naturally expressed in a cell, recombinantly or naturally expressed in cells transplanted into an animal or plant, or recombinantly or naturally expressed in a

transgenic animal. Modulation is tested using one of the *in vitro* or *in vivo* assays described herein.

Cells that have wildtype transporter genes, transporter null mutations, transporter missense mutations, or inactivation of membrane transporters are used in the assays of the invention, both *in vitro* and *in vivo*. Preferably, insect cells are used. Optionally, the cells can be transfected with an exogenous transporter gene operably linked to a constitutive promoter, to provide higher levels of transporter expression. Alternatively, endogenous transporter levels can be examined. The cells can be treated to induce transporter expression. The cells can be immobilized, be in solution, be injected into an animal, or be naturally occurring in a transgenic or non-transgenic animal or plant.

Samples or assays that are treated with a test compound which potentially activates, inhibits, or modulates membrane transporters are compared to control samples that are not treated with the test compound, to examine the extent of modulation. Generally, the compounds to be tested are present in the range from 1 nM to 1000  $\mu$ M. Control samples (untreated with activators, inhibitors, or modulators) are assigned a relative transporter activity value of 100%. Inhibition of transporter activity is achieved when the transporter activity value relative to the control is about 90% (*e.g.*, 10% less than the control), preferably 50%, more preferably 25%. Activation of transporters is achieved when the transporter activity value relative to the control is 110% (*e.g.*, 10% more than the control), more preferably 150%, more preferably 200% higher.

The effects of the test compounds upon the function of the transporter polypeptides can be measured by examining any one of a variety of parameters. For example, parameters such as the affinity, specificity, and inhibition of transport, can be measured. Furthermore, the effects of the test compounds on the transporter protein or mRNA levels, transcriptional activation or repression of a reporter gene can be measured. In each assay, cells expressing membrane transporters are contacted with a test compound and incubated for a suitable amount of time, typically 1-30 minutes. Then, parameters such as those described above are compared to those produced by control cells untreated with the test compound.

In one embodiment, the effect of test compounds upon the function of transporter can be determined by comparing the level of transporter protein or mRNA in treated samples and control samples. The level of transporter protein is measured using immunoassays such as western blotting, ELISA and the like with a transporter specific antibody. For measurement of mRNA, amplification, *e.g.*, using PCR, LCR, or

hybridization assays, *e.g.*, northern hybridization, RNase protection, dot blotting, are preferred. The level of protein or mRNA is detected using directly or indirectly labeled detection agents, *e.g.*, fluorescently or radioactively labeled nucleic acids, radioactively or enzymatically labeled antibodies, and the like, as described herein.

5                   Alternatively, a reporter gene system can be devised using the transporter promoter operably linked to a reporter gene such as luciferase, green fluorescent protein, CAT, or  $\beta$ -gal. After treatment with a potential transporter modulator, the amount of reporter gene transcription, translation, or activity is measured according to standard techniques known to those of skill in the art.

10                   The compounds tested as modulators of transporter activity can be any chemical compound, or biological entity, such as a protein, sugar, nucleic acid or lipid. Alternatively, modulators can be genetically altered versions of the transporter of interest. For example, an antisense construct of a transporter can be used as a modulator.

                    Typically, test compounds will be small chemical molecules and peptides.

15                   Essentially any chemical compound can be used as a potential modulator or ligand in the assays of the invention, although most often compounds that can be dissolved in aqueous or organic (especially DMSO-based) solutions are used. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (*e.g.*, in  
20                   microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs Switzerland), and combinatorial libraries produced by chemical companies.

                    In one preferred embodiment, high throughput screening methods involve  
25                   providing a combinatorial chemical or peptide library containing a large number of potential therapeutic compounds (potential modulator or inhibitor compounds). Such “combinatorial chemical libraries” are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as  
30                   conventional “lead compounds” or can themselves be used as potential or actual insecticides.

                    A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical “building blocks” such as reagents. For example, a linear

combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (*i.e.*, the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (*see, e.g.*, U.S. Patent 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991) and Houghton *et al.*, *Nature* 354:84-88 (1991)). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (*e.g.*, PCT Publication No. WO 91/19735), encoded peptides (*e.g.*, PCT Publication No. WO 93/20242), random bio-oligomers (*e.g.*, PCT Publication No. WO 92/00091), benzodiazepines (*e.g.*, U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, *Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al.*, *J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al.*, *J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al.*, *J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al.*, *Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al.*, *J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (*see* Ausubel, Berger and Sambrook, all *supra*), peptide nucleic acid libraries (*see, e.g.*, U.S. Patent 5,539,083), antibody libraries (*see, e.g.*, Vaughn *et al.*, *Nature Biotechnology*, 14(3):309-314 (1996) and PCT/US96/10287), carbohydrate libraries (*see, e.g.*, Liang *et al.*, *Science*, 274:1520-1522 (1996) and U.S. Patent 5,593,853), small organic molecule libraries (*see, e.g.*, benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent 5,506,337; benzodiazepines, 5,288,514, and the like).

Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.*, 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves commercially available (*see, e.g.*, ComGenex, Princeton, N.J., Asinex, Moscow, Ru,

Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

In one embodiment, the invention provides solid phase based *in vitro* assays in a high throughput format, where the cell or tissue expressing a membrane transporter of the invention is attached to a solid phase substrate. In the high throughput assays of the invention, it is possible to screen up to several thousand different modulators or inhibitors in a single day. In particular, each well of a microtiter plate can be used to run a separate assay against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 100 (*e.g.*, 96) modulators. If 1536 well plates are used, then a single plate can easily assay from about 100- about 1500 different compounds. It is possible to assay several different plates per day; assay screens for up to about 6,000-20,000 different compounds is possible using the integrated systems of the invention. More recently, microfluidic approaches to reagent manipulation have been developed, *e.g.*, by Caliper Technologies (Palo Alto, CA).

Yet another assay for compounds that modulate transporter activity involves computer assisted drug design, in which a computer system is used to generate a three-dimensional structure of a transporter based on the structural information encoded by the amino acid sequence. The input amino acid sequence interacts directly and actively with a preestablished algorithm in a computer program to yield secondary, tertiary, and quaternary structural models of the protein. The models of the protein structure are then examined to identify regions of the structure that have the ability to bind, *e.g.*, ligands. These regions are then used to identify ligands that bind to the protein.

The three-dimensional structural model of the protein is generated by entering the transporter amino acid sequences of at least 10 amino acid residues or corresponding nucleic acid sequences encoding a transporter polypeptide into the computer system. The amino acid sequence of the polypeptide or the nucleic acid encoding the polypeptide is selected from the group consisting of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, and 16 and conservatively modified versions thereof. The amino acid sequence represents the primary sequence or subsequence of the protein, which encodes the structural information of the protein. At least 10 residues of the amino acid sequence (or a nucleotide sequence encoding 10 amino acids) are entered into the computer system from computer keyboards, computer readable substrates that include, but are not limited to, electronic storage media (*e.g.*, magnetic diskettes, tapes, cartridges, and chips), optical



media (*e.g.*, CD ROM), information distributed by internet sites, and by RAM. The three-dimensional structural model of the protein is then generated by the interaction of the amino acid sequence and the computer system, using software known to those of skill in the art. The three-dimensional structural model of the protein can be saved to a computer readable form and be used for further analysis (*e.g.*, identifying potential ligand binding regions of the protein and screening for mutations, alleles and interspecies homologs of the gene).

The amino acid sequence represents a primary structure that encodes the information necessary to form the secondary, tertiary and quaternary structure of the protein of interest. The software looks at certain parameters encoded by the primary sequence to generate the structural model. These parameters are referred to as “energy terms,” and primarily include electrostatic potentials, hydrophobic potentials, solvent accessible surfaces, and hydrogen bonding. Secondary energy terms include van der Waals potentials. Biological molecules form the structures that minimize the energy terms in a cumulative fashion. The computer program is therefore using these terms encoded by the primary structure or amino acid sequence to create the secondary structural model.

The tertiary structure of the protein encoded by the secondary structure is then formed on the basis of the energy terms of the secondary structure. The user at this point can enter additional variables such as whether the protein is membrane bound or soluble, its location in the body, and its cellular location, *e.g.*, cytoplasmic, surface, or nuclear. These variables along with the energy terms of the secondary structure are used to form the model of the tertiary structure. In modeling the tertiary structure, the computer program matches hydrophobic faces of secondary structure with like, and hydrophilic faces of secondary structure with like.

Once the structure has been generated, potential ligand binding regions are identified by the computer system. Three-dimensional structures for potential ligands are generated by entering amino acid or nucleotide sequences or chemical formulas of compounds, as described above. The three-dimensional structure of the potential ligand is then compared to that of the transporter protein to identify ligands that bind to transporter. Binding affinity between the protein and ligands is determined using energy terms to determine which ligands have an enhanced probability of binding to the protein. The results, such as three-dimensional structures for potential ligands and binding affinity of ligands, can also be saved to a computer readable form and can be used for further

analysis (*e.g.*, generating a three dimensional model of mutated proteins having an altered binding affinity for a ligand).

## VI. Insecticidal Preparations

5           The present invention will be particularly useful in the formulation and development of preparations for use in the control of insects. In particular embodiments of insecticide preparations, a composition comprises, as an active ingredient, a test compound, identified in the screening assays described above, that inhibits, activates, or modulates activity of an insect cell membrane transporter, *e.g.*, a proline transporter, a  
10   serotonin transporter, an acetylcholine transporter. Combinations of test compounds that modulate a variety of transporters may also be used in insecticide preparations.

          Bioactive peptides and peptide fragments identified by the assays above as modulating insect cell membrane transporter activity may be formulated in DMSO, or other suitable carrier, to enhance the permeability of the preparation through insect  
15   cuticle. This preparation may then be applied as a spray or delivered into a water source as an insect control strategy.

          Organic molecules identified through the screening assays described above may also be formatted for use as an insect spray, water treatment, or bait.

          In one particular application, a sense, an antisense, or combination of sense  
20   and antisense sequences for one or a combination of the insect cell membrane transporters will be engineered into a virus using standard techniques.

          For use as an insecticide, the virus carrying the membrane transport  
antisense sequence(s) will be formulated according to standard field application  
protocols, and then sprayed, by way of example, onto crops. The occluded virus (OV) is  
25   an example of a suitable virus carrier to be used in these applications, these viruses entering the insect via an oral route and solubilized in the alkaline midgut, thus releasing the embedded virions. The virions will enter the midgut cells and subsequently enter the hemocoel as budded virus (BV), and will then be transported to other tissues via the circulatory system and along the tracheal network via epidermal cells. The infection  
30   process will result in cessation of insect feeding within 5-7 days.

          Application of antisense engineered sequences may be used to provide effective delivery of anti-hormone sequences to pest populations, and be expected to prevent successful insect molting.

In a particular embodiment, viral constructs comprising sense sequences for insect cell membrane transporters are provided, and comprise a sequence essentially as set forth in SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, and 15, which includes the sequences for the proline transporter, acetylcholine transporter, serotonin transporter, glutamate transporter, neurotransmitter transporter encoded by the inebriated gene, and an orphan transporter, a GABA transporter, and a LAT transporter. Viral vectors with each of the sequences are optionally constructed using a baculovirus vector pACUW21. However, other vectors may be used together with these and other of the insect cell membrane transporter encoding sequences, and used in insecticidal preparations.

By way of example, a viral delivery system such as pAcUW2B under the control of a p10 promoter (Stewart et al., 1991) may also be used. Other viral systems, such as those described by Tomalski et al. (1991), and Maeda et al., (1991) also are considered useful in the practice of the present invention, these teachings also being specifically incorporated herein by reference.

The insect cell membrane transporter encoding nucleic acid molecules of the present invention may also be used to transform plants. For example, these coding nucleic acid sequences are optionally used either alone or in combination to transform vegetable and fruit plants. Such may be used as part of a method to enhance insect susceptibility to ecdysis triggering hormone like activity, particularly where it is co-expressed with a suitable gut-permeating agent, such as *Bacillus thuringiensis* or viral proteins.

The test compounds of the invention that modulate the activity of insect cell membrane transporters are optionally formulated in microbial delivery systems for application to plants, animals, or both, as a spray or bait. Examples of these preparations are described below. The various topically active preparations that bind insect cell membrane transporters may be formulated directly into a spray or bait for use in the control of insects by applying to plants and animals.

The modulators, inhibitors, and activators of insect cell membrane transport, and their related homologs for agricultural use will be formulated in a manner appropriate for field application, as sprays, or baits, for release into aquatic environments, and for use in urban dwellings. Treatments for cockroaches, ticks, fleas, termites and other common pests may thus be conveniently and relatively easily provided using the preparations of the present disclosure.

Topically active preparations of the present invention, such as compounds that bind the insect cell membrane transporter, may be formulated for application to

agricultural crops and other plants to control insect populations, for example lepidopteran insect populations. Economic loss to a variety of important agricultural crops, including vegetable crops, cotton, grains, such as corn, wheat and soybeans, attributable to lepidopteran insects, may thus be reduced. Insecticides that include the compounds characterized using the present invention provide alternative approaches for controlling against loss attributable to many varieties of insects without toxicity to other animals and humans.

Modulators of insect cell membrane transporter are effective for inducing developmental effects when injected into the insect. For topically active formulations, a gene encoding the modulator or homolog or biologically active fragment thereof, will be included with a virus and the virus applied to crops (or animals) that are at risk of harboring the insects pests, such as lepidopteran. Alternatively, the effectiveness of these preparations may be further enhanced by including within the virus a neurotoxin that will act to paralyze the virus. Additionally, insect cell membrane transporter binding molecules may be prepared as a topically active insecticide.

#### EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar or desirably different results.

This example describes a protocol for screening compounds for inhibition of insect cell membrane transporters.

Stably or transiently transformed cell lines expressing the transporter in a 24-, 96-, 384- or 1536-well format are exposed for 5-30 minutes to a compound either singly or in a combinatorial form. The exposed cells are analyzed using standard assays for measuring transport capacity. A decrease in transport activity indicates the potential of a compound to inhibit transport.

To screen for compounds affecting the serotonin transporter, CV-1 cells transiently expressing the *Manduca sexta* transporter are plated at ca.  $5 \times 10^5$  in a 24-well tissue culture plate in tissue culture media. The cells are either preincubated with an inhibitor or the inhibitor is added simultaneously with the serotonin. A range of inhibitor concentrations is used. To monitor transport,  $^3\text{H}$ - labeled serotonin is added and the cells are incubated for 15 minutes. The cells are then washed twice with buffer and the uptake of serotonin assessed by scintillation counting.